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(54) Title: <b>METHOD OF RAPID ANALYTE DETECTION</b>			
(57) Abstract <p>A detectable synthetic polymer useful to detect the presence of a microorganism in a test sample is provided. The polymer comprises repeating monomeric units, which incorporate a population of first monomeric units each comprising a binding agent which binds to a microorganism having multiple binding sites for said binding agent and which further incorporate a population of second monomeric units each comprising a detectable label or a binding site for a detectable label.</p>			

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## METHOD OF RAPID ANALYTE DETECTION

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### FIELD OF THE INVENTION

The present invention relates to a method for rapidly detecting analytes including pathogenic agents such as coliform bacteria and fungi.

### BACKGROUND OF THE INVENTION

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The term "coliform bacteria" as used herein refers to a group of bacterial genera made up of *Escherichia*, *Klebsiella*, *Enterobacter*, *Serratia* and *Citrobacter* bacteria. Coliform bacteria tend to be small, gram negative rods that may be either motile or nonmotile. Coliform bacteria have complex membranes that include murein, lipoprotein, phospholipid and lipopolysaccharide (LPS) components arranged in layers. A murein-LPS layer is about 20% of the total bacterial membrane and is responsible for bacterial cell rigidity. The LPS aids in preventing hydrophobic toxins from entering a coliform bacterial cell. The LPS is capable of releasing an endotoxin into a host once coliform bacteria infect the host. In human hosts, the endotoxin is released into the bloodstream.

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Natural competitors of coliform bacteria have evolved secondary metabolites, such as antibiotics, to overcome the LPS defense. Competitors such as soil fungi and Streptomyces as well as gram positive bacteria produce antibiotics. One particular class of lipopeptide antibiotic, polymyxins, are produced by a soil microorganism, *B. polymyxa*.

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The polymyxins are designated by the letters A, B, C, D and E. The polymyxins are toxic to coliform bacteria because these antibiotics bind to the LPS in an outer membrane of the coliform bacteria and disrupt cellular metabolism of a coliform bacterium once translocated to an inner cytoplasmic membrane. In particular, the polymyxins are believed to alter the structure and osmotic properties of the outer membrane. Intact, polymyxin antibiotics are capable of binding to both animal membranes and coliform bacterial membranes.

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Coliform bacteria as well as some fungi may cause infections in

the urinary tract and wounds of a human host. Coliform bacteria may also cause pneumonia, meningitis, septicemia and various gastrointestinal disorders in a human being. It has been estimated that as many as 100,000 deaths in the United States each year are a consequence of gram negative bacteria infections such as coliform bacteria.

In addition to causing disease by direct infection, the endotoxin produced by coliform bacteria produces a variety of effects, such as fever, fatal shock, leukocytic alterations, cytotoxicity, alterations in host response to infections, Sanarelli-Shwartzman reaction and various other undesirable metabolic changes. When coliform bacteria enter the bloodstream of a human being, endotoxic shock plays an important role in weakening the individual. About 30% of individuals with endotoxin in their blood will develop shock. About 40 to 90% of individuals in endotoxic shock die. Endotoxin shock is characterized by an inadequacy of blood supply to vital organs of the host causing cellular hypoxia and metabolic failure. Survival of the host is directly proportional to the length of time needed to recognize the development of bacteremia and adequate treatment of the coliform bacterial infection.

Unfortunately, to date, testing for coliform bacteria, yeast and fungi has been excessively time consuming and labor intensive. while the onset of symptoms from the endotoxin may be exceedingly rapid, laboratory based diagnosis will typically take days. To detect and identify coliform bacteria, it is necessary to expose suspect specimens, such as sputum, tissue, pus, body fluids, rectal swabs or feces to a culture media that will allow the growth of gram negative bacteria but inhibit the growth of gram positive bacteria.

The present techniques used for this type of screening involve aseptic transfer of a sample, streaking the sample having bacterial organisms on agar plates after serial dilution and colony enumeration. This is a laborious and lengthy process requiring a time of at least about 24 to 48 hours for a positive result and substantially longer for a negative result.

Additionally, test solutions containing enteric bacteria, use carbohydrates and acid based indicators to demonstrate carbohydrate

fermentation. Lactose and analogues of lactose are the carbohydrates most frequently used in bacteria testing. This is because the majority of organisms of the genera *Escherichia*, *Enterobacter*, and *Klebsiella*, the enteric organisms present in greatest number in fecal material, ferment this carbohydrate while other intestinal pathogens usually do not. Some media may also contain iron salts for the detection of hydrogen sulfide production to aid in the identification of *Salmonella* colonies. This approach to bacterial testing also requires a lengthy incubation time to grow enough bacteria for testing, at least 24 to 48 hours.

Other analyte tests require an organism to digest a detectable material such as fluorescein. In other tests, an antibody, specific for an antigen on an analyte, is labeled with fluorescein to make a fluorescent antibody.

The Ward et al. patent, U.S. Patent No. 4,687,732, generally describes a detection-visualization composition made up of a visualization polymer. This polymer is in turn made up of detectable visualization units, such as multiple enzymes or labelled polyolefins, which are directly or indirectly bonded together. The visualization polymer is coupled to a detecting agent for the target analyte molecule, such as an antibody, an enzyme, or a strand of DNA receptor protein.

However, a continuing need exists for a sensitive and rapid method to detect extremely small amounts of target biological analytes.

#### SUMMARY OF THE INVENTION

The present invention provides a detectable synthetic polymer comprising repeating monomeric units, wherein a first population of monomeric units each comprises a binding agent, such as an antibiotic, which binds to a microorganism, such as a pathogenic bacterium, having multiple binding sites for said binding agent and wherein a second population of monomeric units each comprises a detectable label, such as a fluorogenic molecule, or a binding site for a detectable label, such as biotin. Preferably, the polymer includes more of said second monomeric units than of said first

monomeric units, i.e., at least a ten-fold excess of the second monomeric units over the first monomeric units.

The present detectable synthetic polymer is employed in an assay method to detect the presence in a sample, such as a liquid sample, of microorganisms, such as bacteria. In the practice of the method, the microorganisms are first immobilized, as on a filter, and contacted with an excess amount of the detectable synthetic polymer. The binding agent, i.e., the antibiotic or a fragment thereof, is preselected to bind to a target population of microorganisms, such as gram positive or gram negative bacteria. Since, preferably, there are multiple binding sites for the binding agent on each target organism, and the number of detectable labels, or binding sites therefore, exceeds the number of binding agents on the detectable polymer, so that the resultant signal is greatly amplified relative to the number of organisms which are initially bound. In some cases, the efficient binding of the binding agents to multiple sites on each organism will enable a detectable complex of, e.g., an antibiotic and a detectable label or a binding site for a detectable label to be visualized with sufficient sensitivity. In other cases, the organisms can be first reacted with an antibiotic coupled to a binding site for a detectable label, and the binding site is subsequently reacted with a labelled compound, such as a polymer comprising a multiplicity of detectable labels, as will be further discussed below.

The present invention provides a method for rapidly detecting an analyte which employs a detectable linear synthetic polymer, or "chemical tag." The chemical tag includes a backbone of one of a synthetic water soluble polymer, a synthetic water insoluble polymer, and analogues and derivatives of these polymers. The chemical tag also includes a plurality of detectable components attached to the polymer backbone via linking moieties. The chemical tag additionally includes one or more binding agents, which are fewer in number than the number of detectable components. The binding agent is capable of binding with the analyte to be detected. Once bound, the analyte is detected by means of the detectable component.

The present invention also provides a detectable synthetic

polymer, or "chemical tag." The chemical tag includes a backbone formed of a synthetic polymer selected from a group that includes synthetic water soluble polymers, synthetic water insoluble polymers, and analogues and derivatives of these polymers. The chemical tag also includes a plurality of  
5 detectable components, each attached to the synthetic polymer backbone via a linking moiety, as well as one or more detectable components linked to the synthetic polymer.

The method of the present invention preferably relies upon formation of a bond between an antibiotic such as polymyxin B and a  
10 lipopolysaccharide (LPS) component of the coliform bacterium such as the endotoxin produced by the bacterium. See, C.R. Raetz, Ann. Rev. Biochem., 59, 129-170 (1990). This bond binds the bacterium or the endotoxin to the chemical tag. Once the bond is formed, the chemical tag can be detected via a detectable label which provides a signal that is amplified relative to the  
15 number of units of analyte due to the presence of multiple detectable sites on each tag molecule. For example, the bound tag may be detected via emissions from multiple fluorescent molecules. The chemical tag can also be detected by the incorporation thereto of molecules such as biotin, which provide binding sites for a detectable label, such as an avidin-enzyme  
20 complex. These molecules can be attached via linking moieties to monomeric units of the water soluble polymer or water insoluble polymer backbone of the chemical tag.

The method for detecting coliform bacteria and endotoxin produced by the coliform bacteria using the chemical tag of the present  
25 invention is a surprisingly rapid and sensitive assay. It is believed that the sensitivity of the chemical tag is such to decrease detectable levels of many analytes by several orders of magnitude over what is now obtainable. However, biochemical and immunochemical methods, presently available detect coliform bacteria down to about 10,000 to 100,000 organisms, the  
30 method of the present invention is capable of detecting organisms in a range of 1 to 1000 organisms. While plating methods take at least 24 hours to produce a positive result, and much longer to produce a conclusive negative

result, the method of the present invention produces a conclusive result within an hour of preparing a sample of a test sample as varied as meat, blood and water.

## 5     DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

      The present invention provides a chemical tag and method for rapid detection of a pathogenic microorganisms, e.g., a bacterium such as a coliform bacterium, a subunit thereof, or endotoxin produced by the coliform bacterium. As used herein, the term "microorganism" includes subunits  
10   thereof, such as membrane fragments and metabolites thereof, such as endotoxins. This method includes providing a biocide which binds to the bacterium, such as an antibiotic, e.g., polymyxin B, as the binding agent. A polymer comprising multiple detectable components is then bound to the antibiotic, i.e., to the polymyxin B component to make a chemical tag that  
15   produces a detectable signal once the polymyxin B component binds to the bacterium or to its endotoxin. The chemical tag is then contacted with the coliform bacterium so that the antibiotic binds to the bacterium to form a complex, and the complex is detected via the signal provided by the detectable component.

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### Synthetic Polymer Backbone

      The chemical tag includes a polymer backbone selected from a group of water soluble polymers that includes an acrylate, a methacrylate, and polyvinyl polymers obtained from vinyl monomers such as polyvinyl alcohol,  
25   and polyvinylpyrrolidinone as well analogues and derivatives of these polymers. The chemical tag backbone may also be selected from a group of water insoluble polymers such as polystyrene as well as analogues and derivatives of polystyrene, such as alkylated polystyrenes.

### 30   Biotin and Biotin-Avidin Complexes

      The chemical tag of the present invention can incorporate multiple biotin molecules covalently bonded to a population of monomers of



the synthetic polymer, via linking moieties, in order to generate a powerful signal. It has been found that derivatives of biotin such as iminobiotin and desthiobiotin are also suitable for use in the chemical tag as binding sites for detectable labels. In one particular embodiment, the covalently bonded biotin molecule provides a reaction site for an avidin component of an

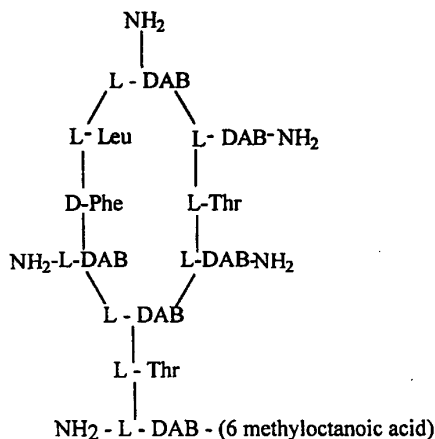
5 molecule provides a reaction site for an avidin component of an avidin-horseradish peroxidase conjugate. In other acceptable embodiments, the biotin molecule may react with another avidin-enzyme conjugate such as an avidin-alkaline phosphatase and an avidin-beta galactosidase conjugate. Because of the strong bonds between biotin and the synthetic monomer and

10 biotin and avidin, the chemical tag of the present invention does not have to covalently bond with the peroxidase or other enzyme. Consequently, enzyme activity is not lost by direct covalent crosslinking chemistry between enzyme molecules and a final detectable signal is maximized at each biotin site.

#### 15 Polymyxin B and Subunits Thereof

Polymyxin B, like the other polymyxins, is a decapeptide having a high percentage of 2,4-diaminobutyric acid (Dab), a fatty acid and a mixture of D- and L-amino acids. Polymyxin B has the following structure:

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In addition to the complete polymyxin B molecule, it has surprisingly been found that the peptide constituent of the polymyxin B which is formed by removal of the 6-methyloctanoic acid moiety, is also usable in the chemical tag of the present invention as the binding component. The polymyxin B molecule is digestible to form the peptide constituent and a lipid constituent that includes octanoic acid. Digestion may be accomplished with a standard proteolytic enzyme. The cyclic nonapeptide constituent may then be easily separated from the lipid constituent and purified by a method such as liquid/liquid extraction. See M. Vaara et al., *Nature*, 303, 526 (1983) and M. Vaara et al., *Antimicrobial Agents & Chemother.*, 24, 107 (1983).

The peptide constituent is a preferred constituent for use in the method of the present invention because the peptide constituent is not toxic to either animal cells or bacteria. The nontoxicity occurs because this constituent cannot bind to a cytoplasmic membrane. However, the peptide constituent does show high avidity and selectivity in binding with the LPS in the outer membrane of gram negative bacteria. This embodiment has been found to be particularly suitable for rapid coliform detection in samples contaminated with eucaryotic cell membranes such as samples of meat, poultry and dairy products. The chemical complex that includes the peptide fragment of polymyxin B is also preferred for detection of endotoxin. A chemical tag made with the peptide constituent includes coupling to a terminal amino group of the peptide fragment to one of biotin or, alternatively, directly to the synthetic polymer.

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#### Other Antibiotic Binding Agents

Additionally, antibiotics selective for gram positive microbes are believed to be suitable binding agents for use in the present invention, such as reistocitin and vancomycin. These antibiotics are glycopeptides that act specifically to block synthesis of peptidoglycan precursors of murein building blocks in the cell walls of gram positive bacteria. Such antibiotics could be used in a manner complementary to the polymyxins to identify each of gram negative and gram positive bacteria. The chemical structure of the

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antibiotics lends the antibiotics either to direct coupling to the chemical tag of the present invention through a single free primary amine group on a peptide moiety, or indirect coupling through a preparation of an avidin conjugate of the antibiotic through the same amino group. A concurrent use of complexes  
5 selected for each of gram positive bacteria and gram negative bacteria could be applicable to field situations in which a knowledge of relative quantities of gram positive and gram negative bacteria are required.

One other group of antibiotics which, it is believed, could serve as binding agents include the aminoglycoside antibiotics. Specific amino  
10 glycoside antibiotics include tobramycin, kanamycin and gentamicin. The aminoglycosides are far less hydrophobic than the polymyxins and are thought to bind the LPS in an outer membrane envelope of gram negative microorganisms. The aminoglycoside antibiotics would then function in a similar fashion to the polymyxins.

15 One additional group of binding agents suitable for detecting eucaryotic microbes such as fungi include the polyene antibiotics such as amphotericin B and nystatin. Each of these antibiotics has a chemical functionality which lends itself to incorporation as a binding agent into the chemical complex of the present invention. This list is presented as  
20 exemplary but not exhaustive of suitable binding agents.

#### Antibiotic-Biotin Complexes

In an embodiment of the chemical tag, the antibiotic, i.e., the polymyxin B or the above-mentioned peptide subunit thereof, is linked to  
25 biotin to form a chemical complex which can in turn be detected with labeled avidin and/or a polymeric tag which can bind to a biotin-avidin complex.

As described, polymyxin B is active as an antibiotic against coliform bacteria. The antibiotic properties of polymyxin B are related to the molecule's capacity to bind the outer bacterial membrane at the LPS  
30 component. About one million binding sites for polymyxin B are available on each coliform bacterium. Thus, polymyxin B and the peptide constituent of polymyxin B have a very high probability of binding to some extent with each

coliform bacterium present in a sample. The present invention takes advantage of this binding capacity to make a chemical tag that may be used to rapidly identify coliform bacteria in extremely small numbers within a particular sample.

5           In this embodiment of the present invention, the biotin molecule is attached to the polymyxin B at one of the amine sites of the polymyxin B molecule. The biotin molecule may also be attached to the amine site of the de-octanoated peptide constituent of PMB. It has surprisingly been found that this attachment does not cripple the capacity of  
10 the polymyxin B to bind to the coliform bacterium. The result is surprising because the amine groups of polymyxin B and the peptide constituent of polymyxin B directly participate in stabilization of the LPS component and outer membrane envelope of the bacterium once the polymyxin B binds to the outer membrane of the bacterium.

15           If biotin is employed as the binding site for a detectable label, once the polymyxin B component or peptide constituent of this complex attaches to a bacterium, the biotin on the chemical tag is detectable by reaction with labelled avidin, i.e., with avidin-horseradish peroxidase or other  
20           avidin-enzyme conjugates such as an avidin-phosphatase or avidin-beta galactosidase. The avidin component of the avidin-horseradish peroxidase binds to the biotin molecule to make a soluble colorless indicator. The colorless avidin indicator may be rapidly visualized with any of a number of colorimetric reagents that react with the horseradish-peroxidase component of the avidin-horseradish peroxidase. These reagents include diaminobenzidine,  
25           aminethylcarbazole and chloronaphthol. These colorimetric reactions require a presence of hydrogen peroxide or organic peroxides as an oxidant. When avidin is bound to a phosphatase, the indicator may be visualized with a naphthol dye. A chemical tag employing this detection system has been found to detect as few as 1000 organisms.

Polymeric Chemical Tag Comprising Antibiotic and Excess Detectable Component

In another embodiment, the chemical tag includes binding molecules that are attached to a synthetic water-soluble polymer backbone.

- 5 Preferably, the chemical tag includes at least one or two polymyxin B components per synthetic polymer in order to optimize binding capacity of the complex of the chemical tag and polymyxin B to the target analyte. Preferred synthetic water-soluble polymers include acrylamide and acrylate polymers.

- The synthetic water-soluble polymer backbone is attached to the
- 10 polymyxin B molecule, preferably with an intermediate linking moiety that is bonded to each of the polymer and the polymyxin B. One preferred intermediate monomer includes an N-hydroxysuccinimide linking moiety, attached to a methacrylic acid monomer. This intermediate monomer is made by preparing an active ester of methacrylic acid. The active ester is prepared
- 15 by reacting the methacrylic acid with a water soluble carbodiimide which is captured as the N-hydroxysuccinimide derivative forming the activated ester. The activated ester undergoes aminolysis by a sufficiently nucleophilic amine group on an incoming peptide such as polymyxin B which forms a stable amide linkage with the methacrylic ester. In one other embodiment,
- 20 polymyxin B is reacted directly with an ethylaminodiethylaminopropyl carbodiimide (EDC) activated methacrylic acid. The activated carboxyl groups of the intermediate synthetic monomer bind with the polymyxin B.

- In this embodiment, the water-soluble polymer backbone of the chemical tag preferably includes more than one biotin molecule. This type of
- 25 chemical tag will have an amplified signal as compared to the chemical tag having polymyxin B attached to a single biotin molecule because of a presence of multiple biotin molecules, each of which can react with avidin-horseradish peroxidase and produce a measurable signal. This chemical tag embodiment has been found to detect as few as 10 organisms.

- 30 One particular chemical tag embodiment preferably additionally includes linking moieties attached to the water soluble polymer backbone. Suitable monomers include electrically neutral water-soluble moieties which are derived from acrylamide and methacrylamide. Each of the linking

moieties is bound to each of the water-soluble polymer backbone and a biotin molecule.

- The complex of the present invention includes another embodiment in which either of the polymyxin B polymer or peptide constituent of polymyxin B is attached to a tag compound having a synthetic water-soluble polymer backbone made up of polymerizable fluorescent monomers and an intermediate amine functional monomer. Preferred fluorescent monomers include N-fluoresceinylacrylamide and sulfonamide obtained from reaction of Sulforhodamine 101 acid chloride (Texas Red™).
- 10 The fluorescent monomers are incorporated into the polymer chain at a minimum stoichiometric ratio of ten monomers to one molecule of the polymyxin B or peptide constituent.

- The fluorescent monomers may be attached to the polymer backbone via reaction with neutral water soluble monomers such as acrylamide and methacrylamide that bind with each of the fluorescent molecules. A preferred intermediate amine functional monomer is N-aminopropyl-methacrylamide. With this fluorescent chemical tag, a significant amplification of fluorescence can be obtained above that obtainable using previous methods. It has been found that this type of chemical tag compound can detect as few as a single organism in a test sample.

- The present invention also includes a chemical tag compound having a water insoluble polymer component that is attached to the polymyxin B molecule. One preferred water insoluble polymer includes a fluorescent polystyrene microsphere of extremely small particle size, such as approximately 0.02 microns in diameter, which is attachable to the polymyxin B molecule. In one particular embodiment, a fluorescent microsphere with an aldehyde surface functionality, such as Fluosphere™, manufactured by Molecular Probes, Inc., of Eugene, Oregon is employed.

- Either of the polymyxin B molecule or the PMB peptide constituent may be attached to the fluorescent microsphere by reductive alkylation to make a chemical tag. This chemical tag avidly binds to coliform bacteria such as *E. coli* and *S. typhimurium*.

Antibiotic-Biotin-Avidin Complex

One other preferred chemical complex embodiment includes a polymyxin B polymer attached to a biotin molecule. An avidin molecule is in turn attached to the biotin molecule. After reaction of this complex with the target microorganism, a biotinylated synthetic polymer such as a biotinylated acrylamide polymer is then attached to the avidin molecule via a biotin site. This chemical tag has been found to detect and visualize a single microbe. This chemical complex is further described in Example 9.

One other preferred chemical complex embodiment includes a first water soluble polymer such as has been described, attached to the polymyxin B and a second biotinylacrylamide polymer in contact with the first polymer. This chemical complex is further described in Example 7.

The use of biotin to make a polymyxin B-biotin conjugate and by attaching multiple biotin molecules to a water soluble synthetic polymer backbone through linking moieties derived from synthetic monomers is useful directly and indirectly, respectively, not only for detecting coliform bacteria but in distinguishing bacteria utilizing lactose from non-lactose utilizing bacteria. In addition to the colorimetric reaction detecting coliform bacteria directly, the peroxidase enzyme component of the avidin-horseradish peroxidase reacts with any hydrogen peroxide that may be generated by the coliform bacterium. Detecting hydrogen peroxide by self-development permits this embodiment of the rapid coliform bacterial detection method to additionally differentiate organisms of the *Escherichia coli* genus species from other non-lactose fermenting gram negative organisms.

Frequently, in water quality monitoring, it is necessary to know not only whether water is contaminated with coliform bacteria but whether the coliform bacteria can utilize lactose. The presence of organisms utilizing lactose implicates the presence of *Escherichia coli* which is a major bacterial organism present in the lower bowel of many mammals.

Existing methods of detecting *E. coli* are cumbersome and excessively time consuming. The methods employ an enrichment culture technique in which a fluorogenic substrate is incubated with an enriched

sample after a 24 hour incubation period. In accord with the rapid method of the present invention, bacteria in a test sample are briefly incubated with an inducer of  $\beta$ -galactosidase, such as isopropylthiogalactoside, which rapidly increases synthesis of  $\beta$ -galactosidase over bacterial basal levels. In the presence of added lactose, glucose and galactose are formed. The bacteria are then treated with either the polymyxin B-biotin tag or the polymyxin B-synthetic water-soluble polymer tag and are immobilized on a microporous filter.

The filter is then treated with a solution of glucose oxidase to convert glucose formed from the bacterial metabolic hydrolysis of the added lactose into gluconic acid and hydrogen peroxide. The hydrogen peroxide reacts in the presence of horseradish peroxidase (HRP) bound to avidin which, in turn, is immobilized on bacterial membranes of bacteria retained on the filter, causing a color reaction. In this manner, the chemical tag and complex of the present invention identify not only coliform bacteria generally but *Escherichia coli* in particular on the membrane filter because of *Escherichia coli*'s ability to metabolize lactose in situ.

In another embodiment, the bacteria immobilized on the filter are induced with the  $\beta$ -gal inducer and the tag is added. The tagged bacteria are then treated with one of bromo or chloro indoyl or bromo- or chloro-naphthol galactoside which is hydrolyzed to galactose and bromo- or chloroindole or naphthol, which are substrates for HRP. An insoluble colored product is formed from the hydrolyzed product by a horseradish peroxidase catalyzed oxidation of the hydrolyzed product.

The methods of the present invention are suitable for use in rapidly detecting coliform bacteria in samples as diverse as drinking water, hamburger and blood. For samples of large volume and mass such as drinking water samples, the present invention includes a kit comprising, packaged together, a filter membrane as well as the chemical tag or complex described. In one embodiment, the filter membrane is a commercial 0.2 micron filter made of a sufficiently transparent material to permit direct insertion of the filter into a device detecting the chemical complex, such as a



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microscope. The filter is employed by the tester to concentrate and collect organisms from a water sample. Once collected, the immobilized bacteria are exposed to the chemical complex and are detected in the manner described.

One other kit embodiment includes a nitrocellulose filter having  
5 antibodies immobilized on the filter. The antibodies may be an element of antisera, either monoclonal or polyclonal, applied to the nitrocellulose filter. The antibodies are directed to specific antigens of a particular type of bacteria. Examples of such antibodies are disclosed by Doyle (U.S. Pat. No. 5,168,063) and R. Szabo et al., *Appl. & Environ. Microbiol.*, 56, 3546 (1990).

10 Once captured and bound on the filter, the bacteria are visualized with the polymyxin B-containing chemical tag. The chemical tag may include biotin on a water soluble polymer backbone with the biotin highly iterated on the polymer. To detect the endotoxin, it is contemplated that one would also use such a filter to collect endotoxin and then tag the  
15 endotoxin with the chemical tag to make the complex described.

Specific examples of the method, chemical tag and complex of the present invention are presented in the Examples that follow. These examples are intended to describe particular aspects of the present invention and are not intended to limit the present invention.

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### EXAMPLES

#### Example 1: Preparation of a polymerizable Polymyxin B Derivative:

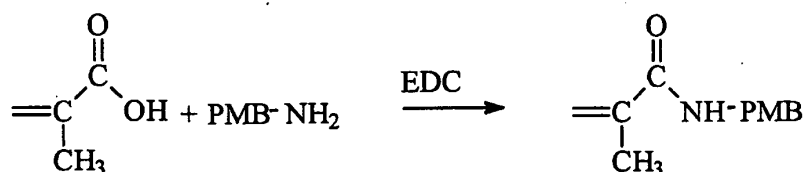
A quantity of 0.0065 g. of methacrylic acid obtained from Sartomar of Exton, Pennsylvania, was dissolved in a quantity of 1  $\mu$ mole, in  
25 5mL 0.004M phosphate buffer having a pH of 6.5 to form a methacrylic acid solution. Once dissolved, a quantity of 0.028 g of ethylaminodiethylaminopropyl carbodiimide (EDC) was added to the methacrylic acid solution. The EDC was obtained from Sigma Chemical of St. Louis, Missouri. The quantity of EDC added was 2.0 equivalents. The  
30 resulting solution was stirred at room temperature for 1 hour to form an active ester.

A quantity of 0.1 g of polymyxin B pentasulfate obtained from

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Sigma of St. Louis, Missouri, was added to a solution of 5mL of 0.05M sodium bicarbonate to form a PMB solution. The PMB solution was added to the active ester to form a reaction mixture. The reaction mixture was incubated for about 4 hours at room temperature to form a PMB acrylamide product. The resulting product was purified by dialysis in a 500 MWCO dialysis membrane obtained from Spectrum Medical Labs of Los Angeles, California, to remove unreacted monomer and water soluble urea product from the product. The product conversion was at least 95% derivative as estimated by thin layer chromatography (TLC). The reaction is as follows:

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#### Example 2: Preparation of a Fluorescent Water Soluble Copolymer.

A terpolymer was prepared by dissolving a quantity of 0.030 g of the PMB acrylamide made in Example 1 into 10 g of water. Once dissolved, a quantity of 1.0 g of acrylamide obtained from Eastman Chemical of Signal Hill, California, and 0.010 g of N-fluoresceinylacrylamide was prepared according to a conventional procedure. One conventional procedure is described in an article of C. Munkholm and D.R. Walt et al., Anal. Chem., v. 58, p. 1427 (1986).

A reaction was carried out in a gas-tight 125mL Ehrlenmeyer flask sealed with a rubber septum to permit replacement of atmosphere within the flask with an inert gas such as nitrogen. The reaction was initiated by addition of 0.005 g of VA 044, bis azo isobutyramidine (WAKO), a water soluble initiator yielding a cationic end group on the polymer. This type of initiator is favored over peroxide type initiators since it results in higher yields

of a linear polymer with less branching. The reaction mixture was statically incubated for sixteen hours at 60°C. The copolymer was purified by both precipitation from acetone and dialysis in a 12,000 MWCO membrane.

A strain of *E. coli*, DH5 $\alpha$ , was obtained from the University of Minnesota. The *E. coli* organisms were maintained on LB agar.

Individual colonies of microorganisms were selected and suspended in a phosphate buffered saline, (PBS) or other buffers conventionally used. One to two colonies of the *E. coli* microbes were suspended in PBS and treated with 0.05mL of 10% solids w/w polymer solution. The microorganisms were then captured by filtration through a 0.22  $\mu$ m Versapor™, manufactured by the Gelman Corp. of Ann Arbor, Michigan, having a 13mm diameter membrane.

The retained material was washed with several volumes of either 10mL PBS or 0.05 M phosphate buffer or 0.5M sodium chloride. The bacterial biomass was visualized with a hand held 366nm U.V. lamp. Negative controls retained on the membrane exhibited no fluorescence. Control samples containing suspensions of *S. aureus* cells obtained from the ATCC also exhibited no fluorescence when stained and subsequently captured by the filter assay technique.

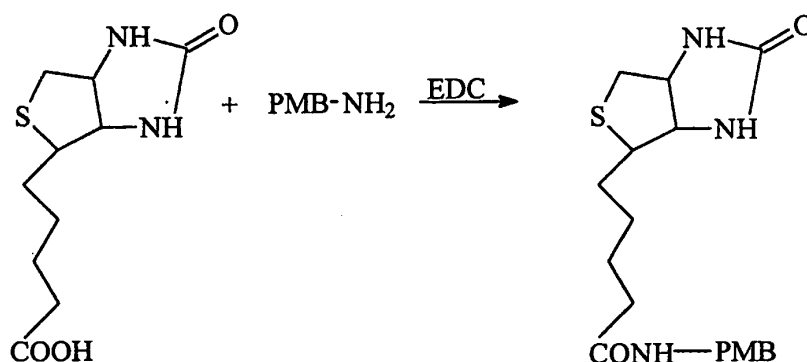
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#### Example 4: Preparation of Polymyxin B Biotin Derivative:

To obtain a sensitivity higher than that shown in Example 3, an ELISA type approach was adopted. For comparison, a direct coupling of polymyxin B to horseradish peroxidase (HRP) was found unsatisfactory in a microbial filter capture assay because of an extremely high background binding of the polymyxin B-HRP conjugate to a commercial microporous membrane.

In the present invention, the polymyxin B was coupled to biotin, the microorganisms were treated in suspension, captured on a membrane filter and then labeled with avidin-horseradish peroxidase conjugate which could then be visualized in situ. The reaction to prepare polymyxin B biotin is as follows:

30



5                    Biotin, obtained from Sigma chemical, was dissolved in  
 a concentration of 0.0185 g, 1μmole, in 5mL absolute ethanol and 10mL of  
 0.02M phosphate buffer having a pH of 7.2. A quantity of 0.028 g of  
 ethylaminodiethylaminopropyl carbodiimide, (EDC) was added in a  
 concentration of 1.3 equivalents with stirring at room temperature for 30  
 10 minutes. A quantity of 0.1 g of polymyxin B in 10mL of phosphate buffer  
 having a pH of 7.2 was added with stirring for 6 hours at room temperature.  
 The product was purified by dialysis against a phosphate buffer in a 500  
 MWCO membrane to remove unreacted materials.

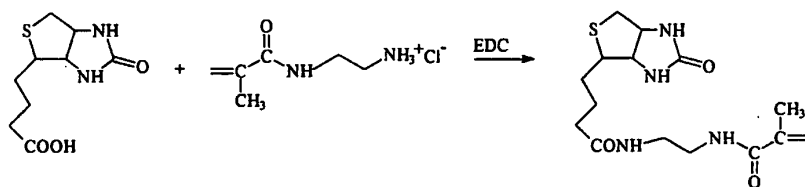
15 Example 5: Evaluation of the Polymyxin B Biotin Probe on E. Coli and  
 Gram Positive Strains:

A single colony of E. coli strain DH5α obtained from the  
 University of Minnesota was selected from an agar plate and suspended in  
 2mL of 0.02M phosphate, 0.15M sodium chloride at a pH of 7.2 to form a  
 20 buffer. The bacterial suspension was treated with 0.05mL, of 0.5 mg  
 PMB-biotin probe as described in Example 4 at room temperature. The cells  
 of the bacterial suspension were captured on a 13 mm microporous membrane  
 disc by filtration, washed with 5mL of the same buffer and treated *in situ* with  
 10μg per mL of avidin-horseradish peroxidase obtained from Sigma Chemical  
 25 in the same buffer containing 5 mg per mL of bovine serum albumin, also  
 obtained from Sigma Chemical.

The membrane was washed with 5mL of the phosphate buffered saline. Cells were visualized by immersing the membrane in 5mL of a diaminobenzidine/hydrogen peroxide reagent obtained as Fast Tabs™ from Sigma Chemical. Negative controls and gram positive cells such as *S. aureus* and *M. luteus* yielded virtually white discs. However, *E. coli* cells present in only a single quantity yielded a dark brown reaction on the test disc.

Example 6: Preparation of a Water Soluble Copolymer of Biotin and PMB

Biotinylmethacrylamide is prepared according to the following reaction scheme:



A quantity of 0.3 g d-Biotin obtained from Sigma Chemical was dissolved in 35mL of absolute ethanol with 20mL of phosphate buffer at a pH of 7.5 and activated by addition of 0.35 g of EDC at a concentration of 1.5 equivalents, along with 8mL of pyridine which was added as a base catalyst. Activation proceeded at room temperature for 30 minutes. After all the reactants were solubilized, 0.22 g of N-aminopropyl-methacrylamide was added to the reaction mixture and stirred for several hours at room temperature. The ethanol solvent was stripped from the mixture by distillation and a residue was washed with several volumes of diluted acetic acid at a concentration of 0.1% v/v.

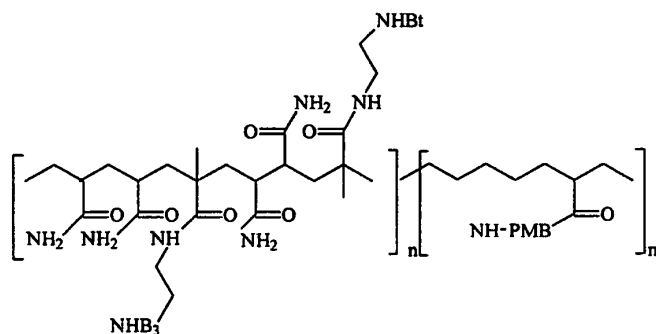
Polymyxin B methacrylamide was prepared according to a similar reaction protocol (Ex. 1). Specifically, 0.0065 g methacrylic acid was dissolved in 5mL of 0.004M sodium phosphate buffer having a pH of 6.5. A quantity of 0.025 g of EDC was added with stirring for 40 minutes at room temperature. A quantity of 0.1 g of polymyxin B pentasulfate was added in 5mL of a 0.05M phosphate buffer having a pH of 7.5 to form a reaction

mixture. The reaction mixture was coupled for several hours and subsequently dialyzed in a 500 MWCO membrane against 2L phosphate buffer.

#### 5 Example 7: Preparation of Biotinylated, PMB Polymer

A quantity of 0.3 g of biotinylmethacrylamide prepared in Example 6 was dissolved in 20mL of ethanol:water in a 50:50 solution to form a biotinylmethacrylamide solution. This solution was added to the 0.1 g of PMB methacrylamide in 20mL phosphate buffer. A quantity of 2.6 g of acrylamide was also dissolved. The entire sample was placed in a 125mL Ehrlemeyer flask and a quantity of 0.007 g VA 044 was added. The flask was sealed under nitrogen at atmospheric pressure and incubated at 55°C for 12 hours to produce a polymer. The polymer obtained was purified by dialysis in a 2000 MWCO membrane against phosphate buffer. A representation of the linear polymer is given the following scheme, wherein n is greater than m, and is determined from the mole ratio of monomers.

Biotinylmethacrylamide + PMB metharylamide + VA 044 + acrylamide →



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It is contemplated that the method and chemical tag of the present invention are suitable for use with binding agents other than polymyxin B to detect analytes other than LPS. For instance, it is believed

that binding agents such as protein A, protein B and other immunogenic as well as non-immunogenic binding agents can be bonded to the chemical tag described. Other binding agents include an antibiotic that binds to an outer membrane of an analyte. In addition to the polymyxin antibiotic, it is

- 5 believed that other antibiotics of the class of cationic oligopeptide antibiotics are suitable binding agents. Other members of this class include the colistins.

#### Example 8. Preparation of Biotinylated Polymer

- A quantity of 0.3 g of the biotinylmethacrylamide prepared as
- 10 described in example 6 was dissolved in 15 mL ethanol water (50:50) and added to 2.0g of acrylamide in 20 mL water. Polymerization initiator, VA 044, 0.010g was dissolved in the reaction mixture. The sample was sealed in a 125 mL Erlenmeyer flask with a tightly fitting rubber septum. The atmosphere was flushed and replaced with nitrogen and incubated statically
- 15 for 18 hours at 55°C. The resultant polymer was purified by dialysis against 0.2M sodium phosphate buffer.

#### Example 9: Evaluation of the Signal Amplification Polymer

- One small colony of *E. coli* DH5 $\alpha$  was suspended in a solution
- 20 of phosphate buffered saline to make a suspension. The suspension was diluted 5,000 and the suspension was then treated with 0.5 mg of PMB-biotin conjugate in accordance with a protocol described in Example 5. Cells captured by the PMB-biotin conjugate were treated in situ with a solution of 2  $\mu$ g per mL of *Streptavidin* obtained from Bethesda Research Laboratories of
- 25 Bethesda, Maryland in a phosphate buffered saline solution with 5 mg per mL bovine serum albumin. The cells, now immobilized, were washed with a phosphate buffered saline that also included a surfactant. The cells were then treated with 5 mL of the polymer of Ex. 8 in phosphate buffered saline having a concentration of 10% solids, by weight and subsequently treated with 10%
- 30 solids, by weight and subsequently treated with 10  $\mu$ g per mL of avidin horseradish peroxidase conjugate in a phosphate buffered saline (PBS) bovine serum albumin (BSA) solution. The cells were then washed and visualized

with diaminobenzidine-hydrogen peroxide tablets.

The samples diluted 5,000 and 150,000 fold were readily visualized over negative controls. Independent plating experiments established that one colony contains approximately 10,000,000 colony forming units  
5 (CFU) by standard plating dilution methods. From this data, it is estimated that the highest dilution enabling an easy visualization is 80 CFU on a membrane.

Based on the density of LPS molecules per cell and molecular weight, 1,000,000 molecules and 1,700 molecules respectively, it is estimated  
10 this quantity of cells to be 0.86 picograms of LPS on the 11 mm membrane. It is believed that higher sensitivity can readily be achieved by any number of adjustable parameters. Conceivably, one to ten bacterial cells could be visualized using this method.

Although the present invention has been described with  
15 reference to preferred embodiments, workers skilled in the art will recognize that changes may be made in form and detail without departing from the spirit and scope of the invention.



## WHAT IS CLAIMED IS:

1. A detectable synthetic polymer comprising repeating monomeric units,  
wherein a first population of the monomeric units each comprises a  
binding agent which binds to a microorganism having multiple binding  
sites for said binding agent and wherein a second population of the  
monomeric units each comprises a detectable label or a binding site for  
a detectable label.
2. The synthetic polymer of claim 1 wherein the polymer includes more  
of said second monomeric units than of said first monomeric units.
3. The synthetic polymer of claim 1 wherein the microorganism is a  
pathogenic bacterium.
4. The synthetic polymer of claim 1 wherein the detectable label or the  
binding site for the detectable label is bound to each of the population  
of said second monomeric units via a linking moiety.
5. The synthetic polymer of claim 4 wherein the detectable label is a  
fluorescent molecule.
6. The synthetic polymer of claim 5 wherein the polymer includes a ten-  
fold excess of second monomeric units.
7. The synthetic polymer of claim 5 which comprises a water-insoluble  
polymeric microsphere.
8. The synthetic polymer of claim 1 wherein the binding site for the  
detectable label is biotin.

9. The synthetic polymer of claim 1 wherein the binding agent is an antibiotic or a fragment thereof.
10. The synthetic polymer of claim 9 wherein the binding agent is  
5 polymyxin B.
11. A detectable compound comprising an antibiotic or antibiotic fragment which binds to a microorganism which has multiple binding sites for said antibiotic or antibiotic fragment, and a detectable label or a  
10 binding site for a detectable label covalently linked to said antibiotic or antibiotic fragment.
12. The compound of claim 11 wherein the binding site for the detectable  
15 label is a biotin molecule covalently linked to said antibiotic or antibiotic fragment.
13. The compound of claim 11 wherein the binding site for the detectable label is an avidin-biotin complex, wherein the biotin molecule is covalently linked to the antibiotic or to the antibiotic fragment.  
20
14. The compound of claim 11 wherein the detectable label is an enzyme-labelled avidin complexed with a biotin molecule, wherein the biotin molecule is covalently linked to the antibiotic or antibiotic fragment.
- 25 15. A method for detecting the presence of microorganisms in a test sample comprising:
- (a) immobilizing microorganisms from said test sample;
  - (b) contacting said microorganisms with an amount of a detectable synthetic polymer which comprises repeating monomeric units,  
30 wherein a first population of the monomeric units each comprises a binding agent which binds to said microorganisms at multiple binding site for said binding agent on each

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microorganism, and wherein a second population of the monomeric units each comprises a detectable label or a binding site for a detectable label, so as to form microorganism-polymer complexes; and

5 (c) detecting said microorganism-polymer complexes.

16. The method of claim 15 wherein the second population of monomeric units is larger than the first population of monomeric units.

10 17. The method of claim 15 wherein the detectable synthetic polymer is essentially linear.

18. The method of claim 16 wherein the binding agent is an antibiotic, which binds to binding sites on a pathogenic microorganism.

15

19. The method of claim 18 wherein the microorganism is a bacterium.

20. The method of claim 19 wherein the microorganism is *E. coli*.

20 21. The method of claim 18 wherein the detectable label is a fluorogenic molecule.

22. The method of claim 18 wherein the binding site for the detectable label is a biotin molecule.

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23. The method of claim 22 wherein the complexes are detected by reaction of the biotin with enzyme labelled avidin followed by a substrate for the enzyme that yields a detectable reaction product.

30 24. A method for detecting the presence of microorganisms in a test sample comprising:

(a) immobilizing microorganisms from said test sample;

- 5 (b) contacting said microorganisms with a detectable compound comprising an antibiotic or antibiotic fragment which binds to a microorganism at multiple binding sites for said binding agent, and a detectable label or a binding site for a detectable label covalently linked to said antibiotic or antibiotic fragments so as to form microorganism-detectable compound complexes; and
- (c) detecting said complexes.
- 10 25. The method of claim 24 wherein the binding site for the detectable label is a biotin molecule covalently linked to said antibiotic or antibiotic fragment.
- 15 26. The method of claim 25 wherein the binding site for the detectable label is an avidin-biotin complex, wherein the biotin molecule is covalently linked to the antibiotic or to the antibiotic fragment.
- 20 27. The method of claim 25 wherein the complexes are detected by reacting them with a polymer comprising multiple biotin molecules, so that the polymer forms a complex with the avidin molecule of the avidin-biotin complex.
- 25 28. The method of claim 25 wherein the complexes are detected by reacting the biotin molecule with an enzyme-labelled avidin.
29. The method of claim 5 wherein the detectable label is an enzyme-labelled avidin complexed with a biotin molecule which is covalently linked to the antibiotic or antibiotic fragment.
- 30 30. A method for detecting the presence of microorganisms which utilize lactose in a test sample comprising:
- (a) immobilizing the microorganisms;
  - (b) contacting said microorganisms with an inducer of  $\beta$ -

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galactosidase to produce  $\beta$ -galactosidase in said test sample;

(c) adding lactose to said test sample, which is converted to glucose;

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(d) contacting said microorganisms with an amount of a detectable compound comprising an antibiotic or antibiotic fragment which binds to said microorganism at multiple binding sites for said antibiotic or antibiotic fragment, and comprising a binding site for a detectable label, said label incorporating a peroxidase enzyme, to form complexes between said detectable compound and said microorganisms;

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(e) contacting said immobilized complexes with glucose oxidase to convert glucose produced by said

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microorganisms to hydrogen peroxide and gluconic acid;

(f) contacting said complexes with a detectable label comprising a peroxidase enzyme which label binds to binding sites on said complexes; and

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(g) contacting said bound complexes an indicator compound and a peroxide, so that a detectable reaction product is formed from said indicator compound in the presence of the peroxidase enzyme.

31. A method for detecting microorganisms which utilize lactose in a test sample, comprising:

25

(a) immobilizing the microorganisms;

(b) contacting the microorganisms with an inducer of  $\beta$ -galactosidase;

30

(c) contacting the microorganisms with an amount of a detectable compound comprising an antibiotic or antibiotic fragment which binds to multiple binding sites on said microorganism, and comprising a binding site

- for a detectable label, said label incorporating a peroxidase enzyme; to form complexes between said microorganisms and said detectable compound;
- 5 (d) contacting the complexes with a bromo- or chloro-indolyl- or naphthyl- galactoside so that galactose and bromo- or chloro-indole or naphthol are formed;
- (e) contacting the complexes with a detectable label comprising a peroxidase enzyme, which label binds to binding sites on said detectable compound; and
- 10 (f) contacting the complexes with an amount of peroxide so that a detectable reaction product is formed from said indole or naphthol.
- 15 32. The method of claims 30 or 31 wherein said microorganisms comprise *E. coli*.
33. The method of claims 30 or 31 wherein said antibiotic is polymyxin B or the nonapeptide fragment thereof.
- 20 34. The method of claims 30 or 31 wherein said binding site for a detectable label is at least one biotin molecule.
35. The method of claim 34 wherein said detectable label incorporating a peroxidase enzyme is an avidin- or streptavidin-peroxidase conjugate.
- 25 36. The method of claim 35 wherein the detectable label is an avidin-horseradish peroxidase conjugate.
- 30 37. The method of claim 32 wherein said binding site for a detectable label is a biotin molecule covalently bound to said antibiotic or antibiotic fragment.

38. The method of claim 34 wherein said detectable compound comprises a plurality of biotin molecules linked to a first population of monomeric units of a linear polymer, and wherein a plurality of said antibiotics or antibiotic fragments is covalently linked to a second
- 5 population of monomeric units of the linear polymer, and wherein the first population is larger than said second population.

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 95/06003

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> IPC 6 G01N33/58 G01N33/569		
According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) IPC 6 G01N C12Q		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO,A,84 04970 (UNIV YALE) 20 December 1984 cited in the application see page 7, line 17 - page 8, line 12 see page 11, line 19 - page 12, line 21 see page 44, line 22 - page 45, line 22 & US,A,4 687 732 ---	1-29
Y	EP,A,0 077 671 (ORTHO DIAGNOSTIC SYSTEMS INC) 27 April 1983 see claims 1,4-6; example 1 ---	1-29
Y	WO,A,91 12282 (FULLER H B LICENSING FINANC) 22 August 1991 see the whole document ---	1-29
Y	EP,A,0 460 385 (FULLER H B LICENSING FINANC) 11 December 1991 see the whole document ---	1-29
-/--		
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "&" document member of the same patent family		
Date of the actual completion of the international search  6 September 1995		Date of mailing of the international search report  20. 09. 95
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016		Authorized officer  Cartagena y Abella,P

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# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 95/06003

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	FR,A,2 295 426 (BLOCK ENGINEERING, INC.) 16 July 1976 see claims 1,3,7,13 ----	1-14
A	EP,A,0 265 127 (HARVEY WILSON ;WILSON MICHAEL (GB)) 27 April 1988 ----	
A	EP,A,0 277 697 (UNIV PITTSBURGH) 10 August 1988 ----	
A	GB,A,2 025 372 (HAMILTON TOOL CO) 23 January 1980 see page 2, line 16 - line 17; claims 1-3 see abstract ----	30
A	WO,A,92 22669 (HYPOGUARD UK LTD) 23 December 1992 see the whole document -----	30

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# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 95/06003

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-8404970	20-12-84	US-A- 4687732	18-08-87
		AU-A- 3150384	04-01-85
		CA-A- 1237369	31-05-88
		DE-A- 3485918	15-10-92
		EP-A, B 0149654	31-07-85
		JP-T- 60501573	19-09-85
EP-A-0077671	27-04-83	US-A- 4434150	28-02-84
		CA-A- 1200760	18-02-86
		JP-C- 1657831	21-04-92
		JP-B- 3024627	03-04-91
		JP-A- 58079162	12-05-83
		JP-A- 3236331	22-10-91
WO-A-9112282	22-08-91	NONE	
EP-A-0460385	11-12-91	US-A- 5142010	25-08-92
		JP-A- 4026669	29-01-92
FR-A-2295426	16-07-76	US-A- 4166105	28-08-79
		US-A- 4169137	25-09-79
		AU-A- 8770775	23-06-77
		CA-A- 1052692	17-04-79
		DE-A- 2557419	24-06-76
		GB-A- 1533410	22-11-78
		JP-A- 51106721	21-09-76
		JP-A- 59131165	27-07-84
		JP-A- 59155760	04-09-84
		NL-A- 7514864	22-06-76
		CA-A- 1054517	15-05-79
EP-A-0265127	27-04-88	WO-A- 8802862	21-04-88
		GB-A, B 2197470	18-05-88
		JP-T- 2500856	22-03-90
EP-A-0277697	10-08-88	US-A- 4870158	26-09-89
		JP-A- 2022295	25-01-90
GB-A-2025372	23-01-80	US-A- 4270743	02-06-81
		CA-A- 1089889	18-11-80

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# INTERNATIONAL SEARCH REPORT :

Information on patent family members

International Application No  
PCT/US 95/06003

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
GB-A-2025372		DE-A- 2909831 JP-A- 55007176	17-01-80 18-01-80
WO-A-9222669	23-12-92	AU-B- 661492 AU-A- 2021192 CA-A- 2110908 EP-A- 0591322 HU-A- 65982 JP-T- 6510426	27-07-95 12-01-93 23-12-92 13-04-94 29-08-94 24-11-94